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MAJ OLSEN ERIC V

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Abstract

Proof-in-concept biosensors were prepared for the rapid detection of *Salmonella typhimurium* in solution, based on affinity-selected filamentous phage prepared as probes physically adsorbed to piezoelectric transducers. Quantitative deposition studies indicated that $\approx 3 \times 10^{10}$ phage particles per cm^2 could be irreversibly adsorbed for 1 h at room temperature to prepare working biosensors. The quality of phage deposition was monitored by fluorescent microscopy. Specific bacterial binding resulted in resonance frequency changes of prepared sensors, which were evaluated using linear regression analysis. Sensors possessed a rapid response time of < 180 s, had a low detection limit of 10^2 cells/ml and were linear over a range of 10^1–10^7 cells/ml with a sensitivity of 10.9 Hz per order of magnitude of *S. typhimurium* concentration. Viscosity effects due to increasing bacterial concentration and non-specific binding were not significant to the piezoelectric platform as confirmed by dose-response analysis. Phage-bacterial binding was confirmed by fluorescence and scanning electron microscopy. Overall, phage may constitute effective bioreceptors for use with analytical platforms for detecting and monitoring bacterial agents, including use in food products and possibly biological warfare applications.

Affinity-selected filamentous bacteriophage as a probe for acoustic wave biodetectors of *Salmonella typhimurium*

Eric V. Olsen[1+], Iryna B. Sorokulova[2], Valery A. Petrenko[3], I-Hsuan Chen[3], James M. Barbaree[1], and Vitaly J. Vodyanoy[2#]

[1]Department of Biological Sciences, Auburn University, 101 Life Sciences Bldg., Auburn, AL 36849
[2]Department of Anatomy, Physiology and Pharmacology, Auburn University, 109 Greene Hall, College of Veterinary Medicine, Auburn, AL 36849
[3]Department of Pathobiology, Auburn University, 252 Greene Hall, College of Veterinary Medicine, Auburn AL 36849
[#]Author for correspondence: Tel. 334-844-5405; Fax 334-844-5388; e-mail: *vodyavi@auburn.edu*
[+]The views expressed in this article are those of the author, and do not reflect the official policy or position of the United States Air Force, Department of Defense, or the U.S. Government.

Key words: biosensor, filamentous bacteriophage, phage display, piezoelectric, quartz crystal microbalance, *Salmonella typhimurium*

1. Introduction

Salmonella typhimurium remains a leading cause of gastrointestinal foodborne illness (Preliminary foodnet data, 2003) and a potential weapon for bioterrorism (Khan et al., 2001). The ability to rapidly identify this biological agent and other pathogens, regardless of their contamination origin, is an important consideration of any comprehensive strategic plan to maintain safety and security of the public food supply (Progress report to Secretary Tommy G. Thompson, 2003). Current research initiatives to replace conventional methods of food analysis that are slower, labor-intensive, and cost-inefficient include real-time, deployable threat agent detectors based on biosensors (Alocilja and Radke, 2003). The majority of rapid detection biosensors described in the literature for *S. typhimurium* have utilized antibodies as bioreceptors (Kaspar et al., 2000; Skládal, 2003). Antibodies can be extremely narrow (monoclonal) or broad (polyclonal) in their specificity and generally have very high affinities (picomolar). In the production of quartz crystal microbalance (QCM)-based immunosensors, they are immobilized to the substrate via their hydrophobic F_c fraction, with the antigen binding F_{ab} fraction being oriented outward to capture the target. However, while sensitive and selective, antibodies possess numerous disadvantages for use as biodetectors in food products or as diagnostic bench-top sensors. These disadvantages include high cost of production, low availability, high fragility, lack of reusability, and the need for laborious immobilization methods to sensor substrates (Petrenko and Vodyanoy, 2003).

A resilient alternative to antibodies is filamentous phage affinity-selected from a landscape library for specific, selective binding to a target biomolecules (Sorokulova, et

al., 2005). The Ff class of filamentous bacteriophage can tolerate the incorporation of foreign DNA through recombinant modification – expressing the foreign peptides on the surface of the virion for presentation to a complementary target, such as cell receptors on the outer membrane of *Salmonella*. In this way they can act as antibody substitutes, possessing distinct advantages over their natural counterparts including durability, reusability, stability, standardization, and low-cost production, while achieving equivalent specificity and sensitivity (Petrenko and Smith, 2000). The use of phage display to prepare phage as antibody substitute probes to overcome the weak points of antibodies has been discussed (Petrenko and Vodyanoy, 2003).

Numerous phage applications have been proposed (Smith and Petrenko, 1997), including the detection of bacteria (Petrenko and Sorokulova, 2004). Previously, we demonstrated that affinity-selected landscape phage probes for *S. typhimurium* possess the specificity, selectivity and affinity of monoclonal antibodies and can be utilized as probes for the detection of *S. typhimurium* (Sorokulova et al., 2005). Phage-based electronic-based biodetectors for bacteria have not been described in the literature, although Petrenko and Vodyanoy (2003) have discussed the potential of phage in conjunction with the QCM for proof-in-concept detection of threat agents. In addition to previously mentioned advantages over antibodies, the outer coat protein structure of filamentous phage appears to be highly amenable to simple immobilization through physical adsorption directly to the gold surface of the QCM electrode, thus providing another engineering advantage while maintaining peak biological functionality. As proof-of-concept, we prepared biosensors for the rapid detection of *S. typhimurium* based

on affinity-selected filamentous bacteriophage immobilized to acoustic wave transducers by physical adsorption.

2. Materials and methods

2.1. Microorganisms

2.1.1. Bacteriophage

Filamentous phage as specific, selective probes for *S. typhimurium* (clone E2 – displaying foreign peptide VTPPTQHQ used in this work) were derived previously from a landscape f8/8 phage library (Petrenko et al., 1996) through affinity selection procedures described in Sorokulova et al. (2005).

2.1.2. Bacteria

S. typhimurium (ATCC 13311) obtained from the American Type Culture Collection (Rockville, MD) was confirmed for identity, propagated, and maintained as described in Sorokulova et al. (2005).

2.2. Test solutions

The functional performance of biosensors was evaluated with bacterial suspensions prepared from an overnight culture of *S. typhimurium* in an orbital shaker–incubator (37 °C, ≈ 200 rpm). The overnight culture was washed (3×) by

centrifugation (5500 rpm, 15 min, 10 °C) in 20 ml sterile phosphate-buffered saline (PBS: 0.15 M NaCl, 5 mM NaH_2PO_4, [pH 7.0]) with final resuspension in 2 ml PBS. An aliquot (ranging from 125 µl to 600 µl depending upon growth) of the culture was diluted with 4 ml 0.5% (v/v) Tween20 in 1X TBS (50 mM Tris–HCl, 0.15 M NaCl, pH 7.5) to prepare a final suspension of OD 0.8_{620}. The suspension was serially diluted with PBS to prepare bacterial suspensions ranging from approximately 10^1 to 10^7 cells/ml. Bacterial cell counts were confirmed through standard plate count of the three most dilute cell suspensions giving 30 – 300 colonies. Plate counts were performed in triplicate, averaged, and multiplied by the dilution factor to derive CFU count per milliliter. All test solutions were prepared the day of biosensor testing and maintained on ice or at 4 °C until use, then brought to room temperature.

2.3. Bacteria binding measurements

The Maxtek acoustic wave device (AWD), accompanying quartz resonators, and binding measurement procedures used in deposition experiments and sensor evaluations have been described (Olsen et al., 2003; Pathirana, et al., 2000). Monitors were calibrated prior to use. All experiments were conducted at room temperature. All components used in deposition experiments were contained within an Atmosbag™ isolation chamber (Sigma-Aldrich) inflated with purified nitrogen gas to prevent airborne contamination of the resonator.

The AWD was interfaced with a data-processing unit, where sensor response output in the form of frequency or voltage changes were recorded. Response data from

experiments was analyzed using Microcal™ Origin® 6.0 (Microcal Software, Northampton, MA). For mass deposition, graphs depicting frequency change as a function of time were prepared. The frequency difference between beginning (prior to deposition) and ending (following deposition) steady–state levels of masses was determined from these graphs. For biosensors, a cumulative graph of frequency or voltage changes as a function of time was plotted for all bacterial suspensions tested on that sensor. Visual inspection of this graph allowed estimation of steady-state sensor responses. Generally, steady-state was achieved within several hundred seconds following application of the test suspension, but in order to maintain data congruency between all sensors was considered to be the last two minutes of each seven-minute test suspension incubation. From these last two minutes, an average steady-state frequency response for each test suspension applied to a sensor was determined statistically (120 data points), then plotted to derive a graph of the mean steady-state responses of the sensor as a function of the bacterial test suspension concentration. Linear regression was applied to the data points to determine correlation coefficient, slope, and significance. Biosensor results were considered statistically different when the p–value was less than 0.05 (95% confidence interval).

2.4. Microscopic analysis of sensors

Confirmation of phage immobilization and phage-bacteria binding at the sensor surface was conducted by fluorescent and scanning electron microscopy (SEM). Labeling of the phage has been previously described (Sorokulova et al., 2005). *S.*

typhimurium cells were fluorescently labeled (Alexa Fluor 488 Protein Labeling Kit, Molecular Probes, Eugene, OR) by adding a bacterial suspension (1 ml; 10^7 cells/ml in PBS) to a vial of reactive dye, then incubating 1 h at 4 °C. Cells were washed (3×) with 20 ml PBS by centrifugation (3500 rpm, 10 min, 4 °C) prior to use. Fluorescence microscopy arrangement consisted of an operational QCM apparatus with installed resonator viewed under a Nikon (Tokyo, Japan) ECLIPSE E800 fluorescent microscope equipped with 100W Hg lamp, and 4× (NA 0.13), 40× (Plan Fluor DIC M, NA 0.75), 60× (Plan Apo, NA 1.40) and 100× objectives (Plan Apo, NA 1.40). Digital images (500 – 550 nm) were captured using a SPOT RT Slider CCD video camera (1520 × 1080 pixel optical resolution) (Diagnostic Instruments, Inc., Sterling Heights, MI) with exposure settings: red – 1.3 s, green – 2.02 s. Images were transferred to a data processing unit (Dell Corp., Round Rock, TX) for storage and processing using SPOT Advanced software (Ver. 3.5.9 for Windows 2000; build 6 August 2003, Diagnostic Instruments) and Adobe® Photoshop® (Ver. 5.0LE, Adobe Systems, San Jose, CA).

Sequential photos of phage adsorption to resonators were taken in 20 s intervals for up to 2 h. Following phage adsorption for a specified duration, the resonator was removed from the sensor probe using clean forceps, rinsed of excess phage by immersion in degassed water (3×), and examined under fluorescent oil immersion (× 1000). A negative control, consisting of a new, clean resonator treated with Alexa 488 fluorescent dye, was incorporated into the study for comparison.

For SEM, previously assayed biosensors were air-dried at room temperature for 24 h in a sealed petri dish by elevating them over a small layer of desiccant using wooden applicator sticks. A resonator incorporating only phage was prepared in the same manner

as a control. The dried sensors were mounted onto aluminum stubs with carbon adhesive tape then sputter-coated at 0.02 mbar Ar gas pressure with a 60:40 Au/Pd mixture at 30 mA for 1.5 min. Sensors were examined using a Zeiss DSM 940 SEM (Thornwood, NY) at 10 kV. Micrographs of the specimens were captured digitally using a Digital Image Transfer Recognition Program (Zeiss), and processed with Adobe Photoshop 5.0LE.

3. Results and Discussion

3.1. Phage deposition to resonators by physical adsorption

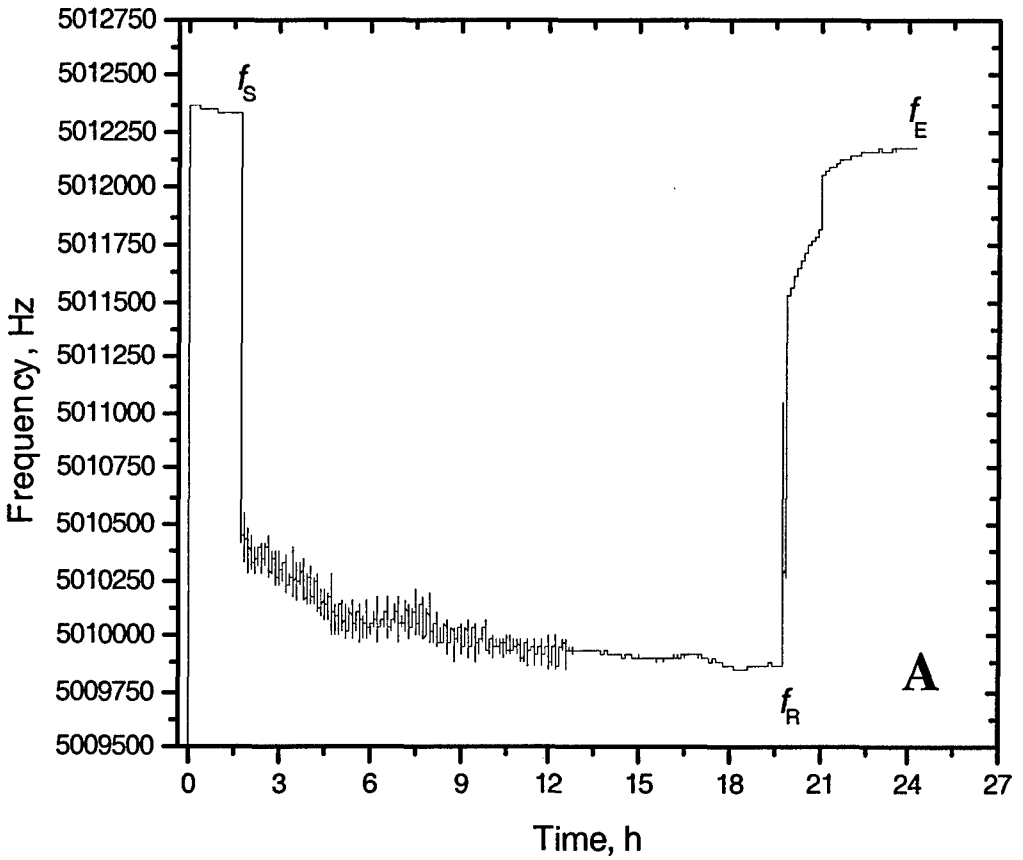
An initial phage deposition study based on the "dip and dry" gravimetric procedure of Prusak-Sochaczewski and Luong (1990) was conducted to determine the feasibility of physically adsorbing filamentous phage to resonators and if successful, the optimal conditions of time and concentration necessary for the preparation of biosensors capable of detecting *S. typhimurium* under liquid conditions. Dip and dry refers to measurement of the change in the resonant frequency, Δf, of a dry resonator prior to and after deposition of mass. According to theory (Sauerbrey, 1959), a mass (attributed here to the mass of phage), m, deposited to the active area of the sensing electrode results in a decrease of the resonator's oscillation frequency, the total value of which can be solved for using Sauerbrey's (1959) equation as follows, assuming that the mass creates a rigid, uniform film that does not slip and has the same acousto-elastic properties as quartz:

$$\Delta f = -Cf(\Delta m) \qquad (1)$$

where, Δf is the observed change in frequency (Hz) of the resonator under oscillation at its fundamental frequency due to mass loading, Cf = sensitivity factor of the resonator in Hz/ng/cm^2, and Δm = change in mass per unit area in g/cm^2. The sensitivity factor (Cf) of Maxtek's 5 MHz AT-cut resonator is 0.0566 Hz/ng/cm^2 at 20 °C, and takes into account the harmonic number at which the resonator is driven, resonant frequency (Hz) of the fundamental mode of the resonator, and the density and effective piezoelectrically stiffened shear modulus of the quartz substrate (RQCM Operation and Service Manual, 2003).

Using this approach, the resonator acts as a gravimetric sensor, much like a very sensitive nanobalance. A representative graph demonstrating phage adsorption as a function of time is shown in Fig. 1A. The graph depicts the steady-state oscillation of a dry, clean resonator at resonance prior to the application of 1 ml of diluted stock phage E2 in suspension (6.7×10^{10} virions/ml) (f_S), followed by an 18 h incubation period at room temperature, removal (f_R) of the phage suspension and washing with degassed water, and finally drying, with a subsequent return to steady-state resonance (f_E). The resulting frequency change, Δf, measured as a decrease, $f_S - f_E$, was -161 ± 0.5 Hz, indicating that phage adsorbed to the resonator. This is contrasted by a control (Fig. 1B) consisting of a clean resonator interrogated with degassed water only, which resulted in no frequency change ($f_S - f_E = 0 \pm 0.5$ Hz).

Figure 1A.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5010250
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Frequency, Hz
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21
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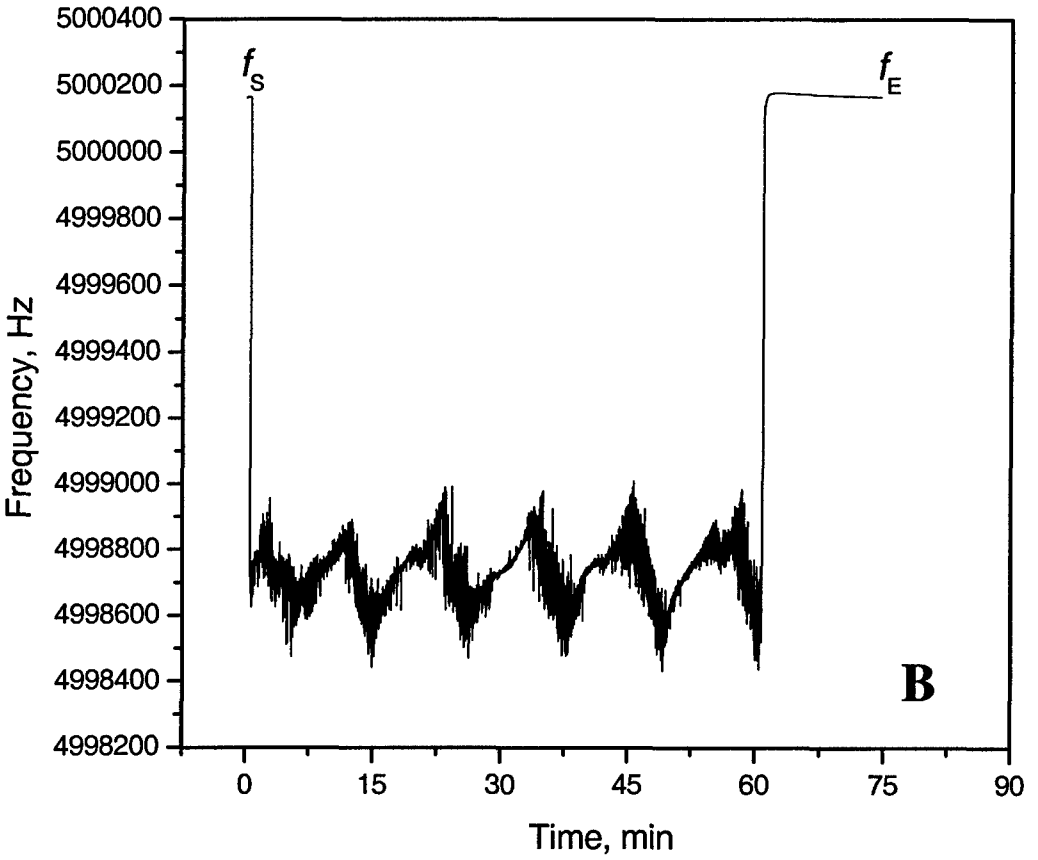
Time, h
f_S
f_E
f_R
A

Figure 1B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4999600
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4999000
4998800
4998600
4998400
4998200
Frequency, Hz
f_S
f_E
B
0
15
30
45
60
75
90
Time, min

Resonance frequency changes due to phage adsorption were determined for periods up to 24 h (1440 min) (Table 1). When the quantity of adhered phage is graphed as a function of time (Fig. 2) and a sigmoidal curve is fitted to the experimental data points, a strong relationship is evident ($R^2 = 0.98$). Extrapolation of the curve at the 120 min time point yields Δf of approximately – 150 Hz. This represents a 92% total frequency change in relation to the maximum frequency change observed at 24 h (–163 Hz), indicating that the majority of phage appears to adsorb within the first few hours after deposition is commenced.

Table 1

Quantity of phage physically adsorbed to resonators as a function of time.

Incubation (min)	$-\Delta f$ (Hz)	Δm (ng)[d]	Phage adsorbed (virions)[e]
20	45[a]	795	1.80×10^{10}
40	60[b]	1065	2.41×10^{10}
60	92[c]	1625	3.68×10^{10}
1080	136	2402	5.45×10^{10}
1440	163	2880	6.50×10^{10}

[a]Mean average of 5 experiments, SD = 31.1 Hz.

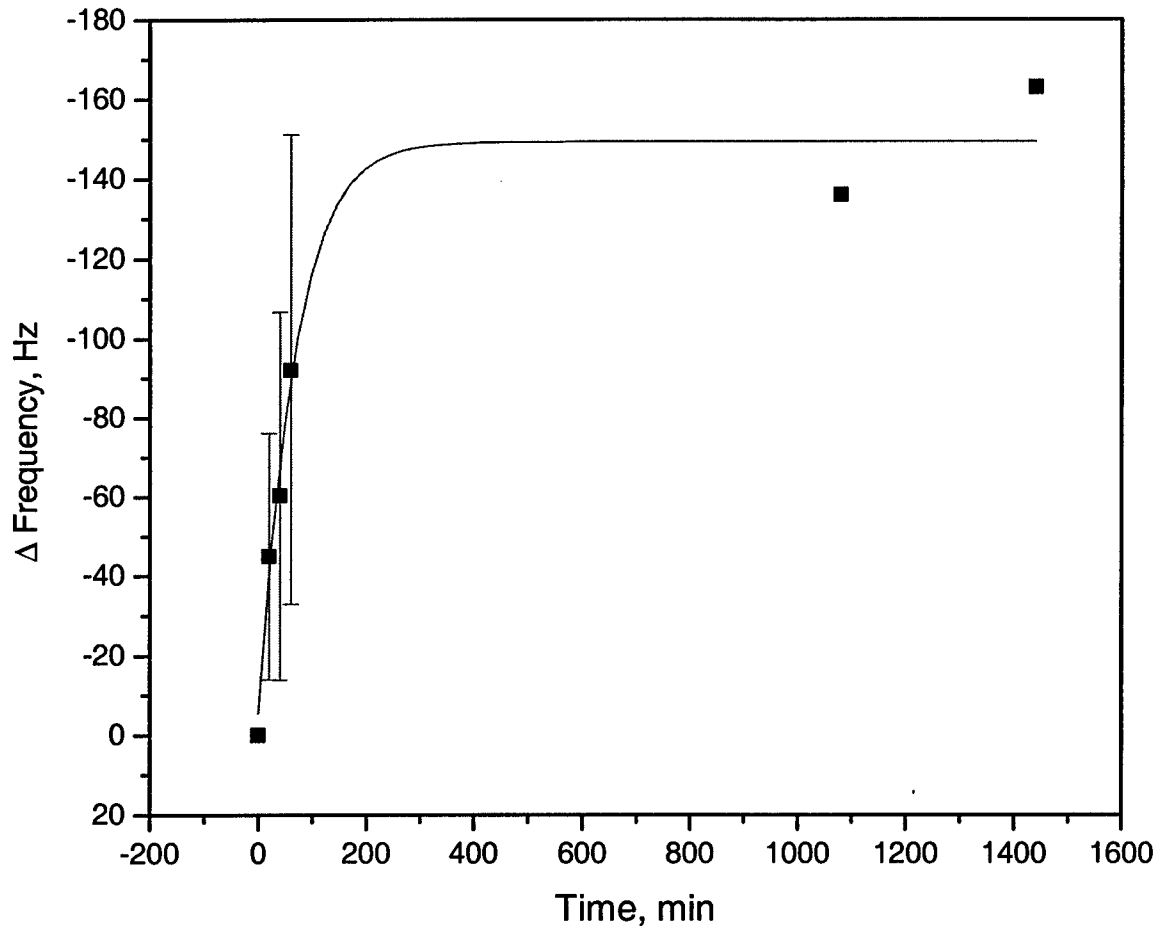
[b]Mean average of 3 experiments, SD = 46.5 Hz.

[c]Mean average of 4 experiments, SD = 59.2 Hz.

[d]Adsorbed phage mass as determined by Sauerbrey equation, $\Delta f = (0.0566)(\Delta m)$.

[e]Quantity of phage deposited as calculated from $\Delta m / m_v$, where the mass of a single virion (m_v) is 2.66×10^7 dal/6.023×10^{23} dal = 44.1×10^{-9} ng.

Figure 2


-180
-160
-140
-120
-100
-80
-60
-40
-20
0
20
Δ Frequency, Hz
-200
0
200
400
600
800
1000
1200
1400
1600
Time, min

The quantity of phage, in virions, can be calculated from the total adsorbed biomass, Δm, if the mass of a single recombinant fd-tet phage is estimated at 2.66×10^7 daltons, based on 4000 pVIII outer coat proteins each containing 55 amino acids with a total molecular weight 2.35×10^7, and DNA with a molecular weight of 3.04×10^6. As shown in Table 1, the total number of phage particles deposited to the active area of the sensing electrode ranged from $1.8 \times 10^{10} - 6.5 \times 10^{10}$ virions as a function of the substrate's exposure time (20 min – 24 h, respectively) to phage in suspension.

Phage adsorption to the sensing electrode of the resonator was confirmed in real-time by fluorescence microscopy for periods up to two consecutive hours (Fig. 3). Visual observations indicate that phage deposition may be affected by several factors in addition to the attractive force between the phage and gold (electrode of the resonator), including sedimentation, refractory movements of the phage in suspension due to resonator perturbations, and the tendency of phage to aggregate in solution, resulting in decreased quantity and quality of deposition when large bundles deposit outside that portion of the sensing electrode where piezoelectricity, and thus frequency changes associated with the resonator, is active. This statement is consistent with large standard deviations in 20, 40, and 60 min deposition experiments (Fig. 2).

Figure 3A.

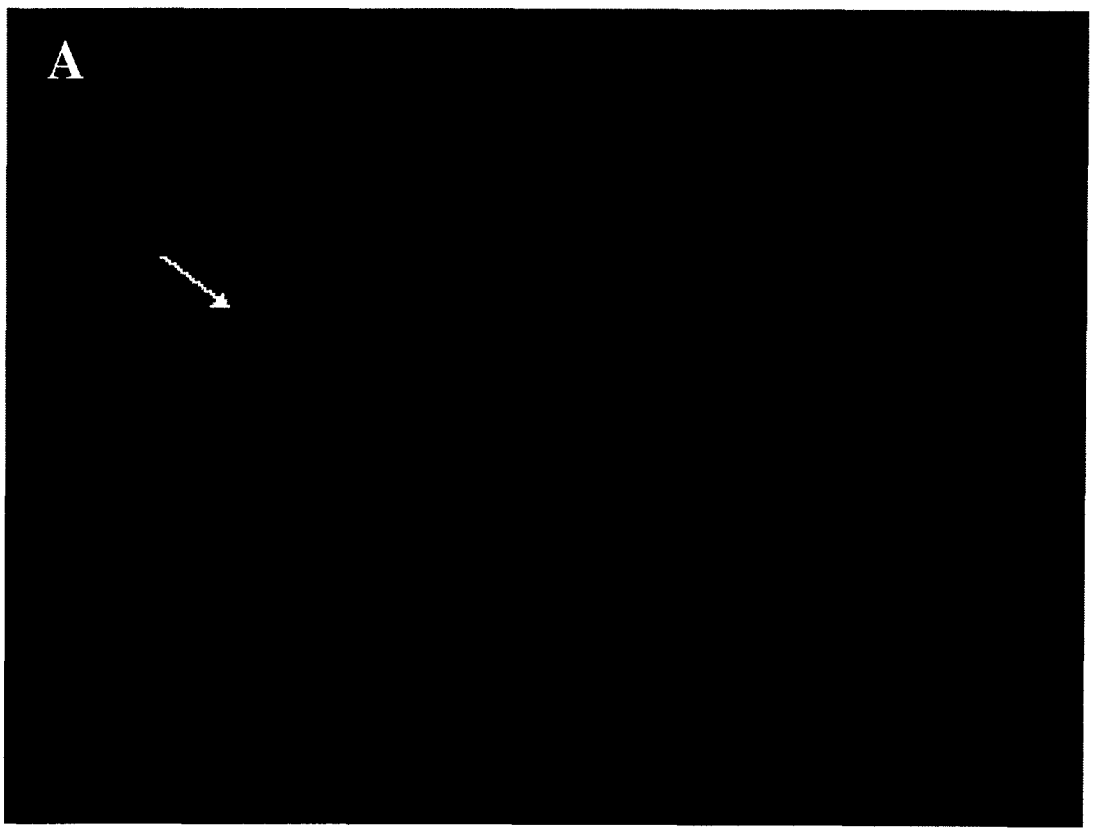
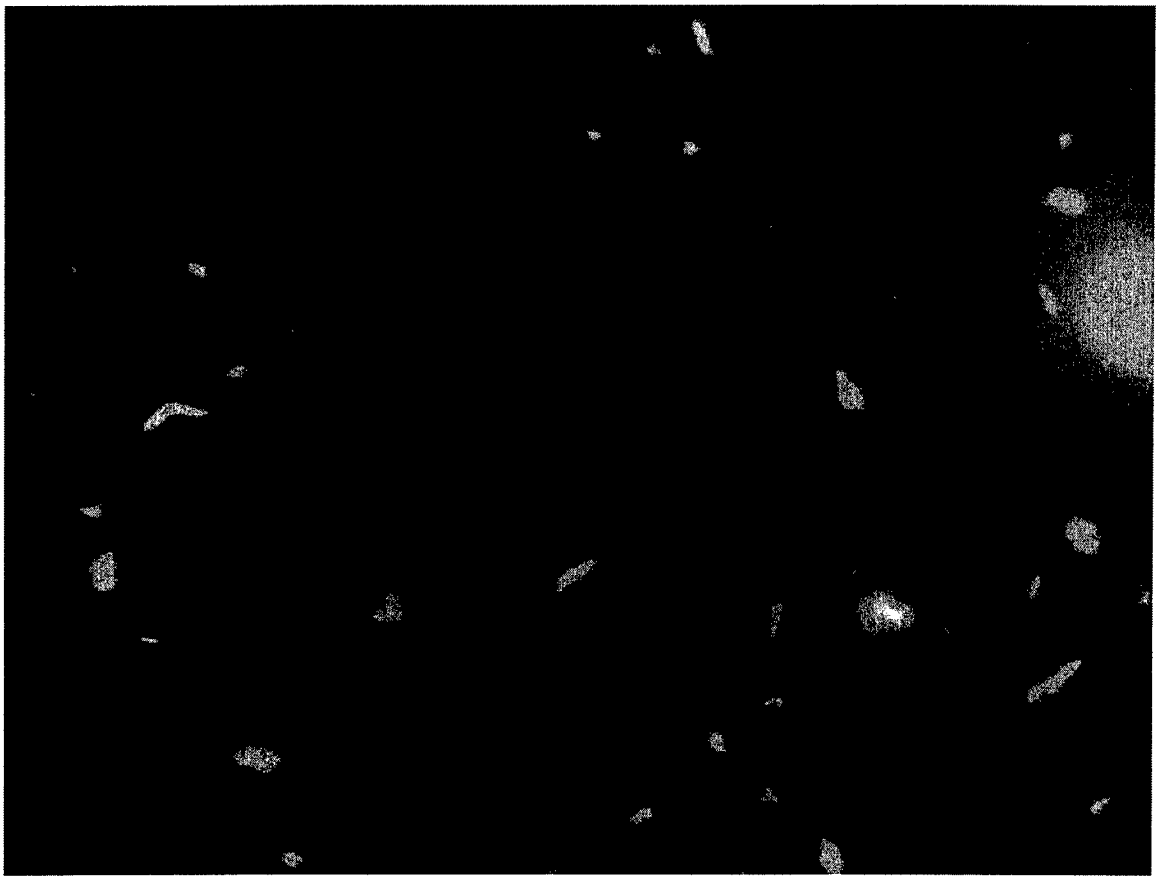


Figure 3B.



Quantitative deposition data together with visual observations suggests that lateral phage aggregates or "bundles," rather than individual particles, might be the prevalent state of deposition to the electrode if one considers the size of individual phage particles and the area of the electrode available for deposition. If the dimensions of a single recombinant fd-tet virion are estimated at 10 nm (diameter) × 1,300 nm (length), then the approximate electrode surface area that would be occupied in a space-filling model by a single, laterally adsorbed phage particle would be 13,000 nm^2 or 1.3×10^{-14} m^2. The active area of the sensing electrode is equal to 34.19 mm^2 or 34×10^{-6} m^2 of the upper sensing electrode (RQCM Operation and Service Manual, 2003). Given these dimensions, the estimated total number of adsorbed phage particles that could occupy the active sensing electrode surface area, if laterally aligned one layer thick, is 34×10^{-6} $m^2/1.3 \times 10^{-14}$ $m^2 = 2.6 \times 10^9$ virions. Experimental data (Table 1) shows that the amount of phage deposited within only 20 minutes exceeds this quantity five-fold. This would seem to indicate that either five or more unidirectional aligned layers of individual particles, or more likely large bundles of phage particles that bind to each other as aggregates in solution, are deposited. An alternative explanation is that phage is adsorbed on-end; that is, the particle attaches by its tip hydrophobically through charge differences or via cysteine residues in minor coat proteins, forming Au-sulfide bonds. According to the space-filling model of Yaglom (1972), packaging occurs most economically when discs are arranged as a hexagonal lattice. Therefore, if cylindrical phage particles are attached by the tip then the density of virions will be much larger compared to the oblique orientation. In this case the density of filling (f) is equal to:

$$f = \pi\sqrt{3}/6 \approx 0.907 \quad (2)$$

The surface occupied by a single vertical phage particle would be estimated as:

$$S_v = \pi d^2/4 = 3.14 \times 10^2/4 = 78.5 \text{ nm}^2 = 7.85 \times 10^{-17} \text{ m}^2, \quad (3)$$

and the number of phage particles covering the active area of electrode would be equal to:

$$N_v = 0.907 \times S_{active}/S_v = 0.907 \times 34 \times 10^{-6} \text{ m}^2/7.85 \times 10^{-17} \text{ m}^2 = \quad (4)$$

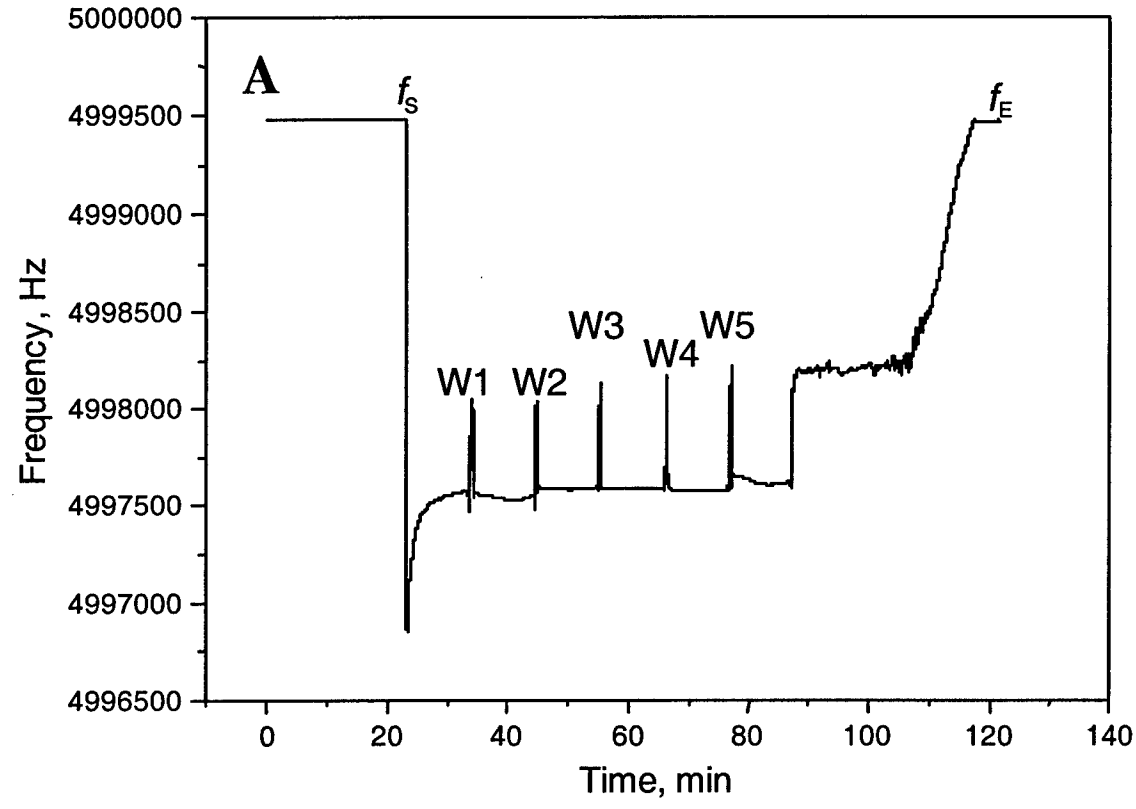
$$0.907 \times 4.33 \times 10^{11} = 3.92 \times 10^{11} \text{ virions.}$$

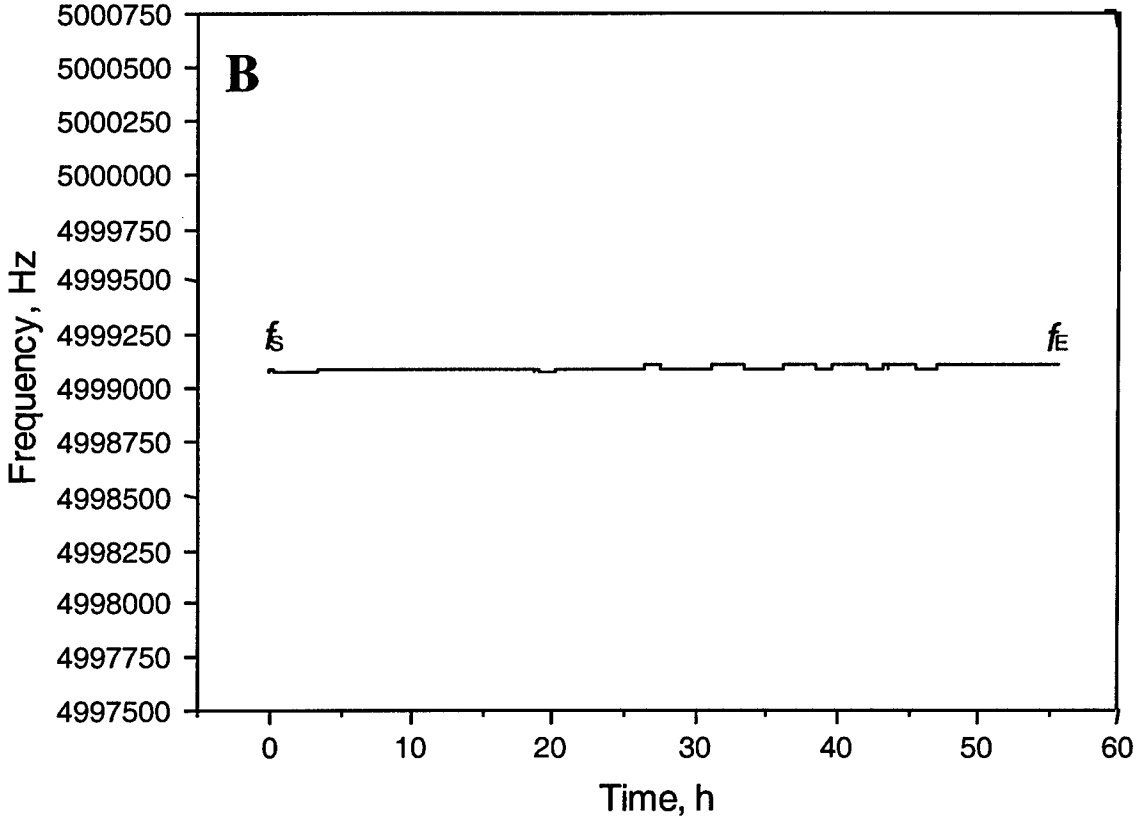
Overman et al., (2004) have demonstrated salt- and concentration-dependent associative properties of phage aggregates, resulting in different physical alignments (unilateral or non unidirectional) based on molar concentration of salt and virion concentration in solution. Thus, further experimentation with salt-virion proportions and concentrations may reveal different physical adsorption properties.

Deposition of phage to the sensing electrode was characterized by strong, non-reversible binding under aqueous conditions, as washing with numerous changes of water (Fig 4A.) or extended immersion in water (Fig 4B.) under operational conditions for durations up to 55 h resulted in no measurable loss of adsorbed phage from the surface of the resonator. While the exact nature and strength of the attractive force between phage and resonator was not determined, possible explanations for protein physical adsorption

to gold include hydrophobic bonding, weak H+ bonding, and Van der Walls forces, along with covalent bonding through Cys-SH groups mentioned above.

Figure 4A and 4B.





3.2. Biosensor preparation and testing

Based on phage deposition studies with concurrent visual observations, a period of 1 h was selected for the preparation of biosensors by physical adsorption. For each sensor prepared, 1 ml of diluted phage (6.7×10^{10} virions/ml with PBS) was incubated on the resonator for 1 h at room temperature, ensuring total immersion of the sensing electrode. Following this period, the phage solution was gently removed then the resonator was washed (3×) by immersion in 25 ml PBS with gentle rocking and blocked with bovine serum albumin (BSA) (1 μg/ml for 1 h at 4 °C). The resonator was again washed (3×) with PBS then tested with a logarithmic series of *S. typhimurium* test suspension as previously described (Olsen et al., 2003; Pathirana et al., 2000). Fig. 5A depicts typical frequency changes resulting from bacterial binding as a function of increasing logarithmic concentrations of free bacteria in suspension. These changes are hypothetically due to both mass changes associated with binding bacteria and viscoelastic changes resulting from phage-bacteria interaction at the solid/liquid interface (Pathirana, et al., 2000). For each concentration, the sensor quickly comes to steady-state equilibrium within several hundred seconds following specific phage-bacteria binding. When the mean values of steady-state frequency readings are plotted as a function of bacterial concentration (Fig. 5B), and a linear line fit, a high dose-response relationship is evident ($R = -0.98$, $p < 0.001$) with the biosensor being linear over six decades of bacterial concentration. The sensitivity of the biosensor (−10.9 Hz), measured as the slope of the linear portion of the dose-response, was vastly greater than the established background ($< \pm 1$ Hz – data not shown). The lower limit of detection based on the dose

response curve was estimated at 100 cells/ml, well below the infectious dosage reported for *Salmonella*-induced gastroenteritis. These results are very comparable with our previous observations for *Salmonella* biosensors prepared with polyclonal antibodies (Olsen et al., 2003; Pathirana et al., 2000). Viscosity effects on the piezoelectric platform due to increasing bacterial concentration and non-specific binding are insignificant as confirmed by dose-response curves (Figs. 5C and 5D). Visual observation by fluorescent and scanning electron microscopy (Fig. 6) confirmed attachment of bacteria to the surface of the resonator coated with filamentous phage.

Figure 5A. and 5B

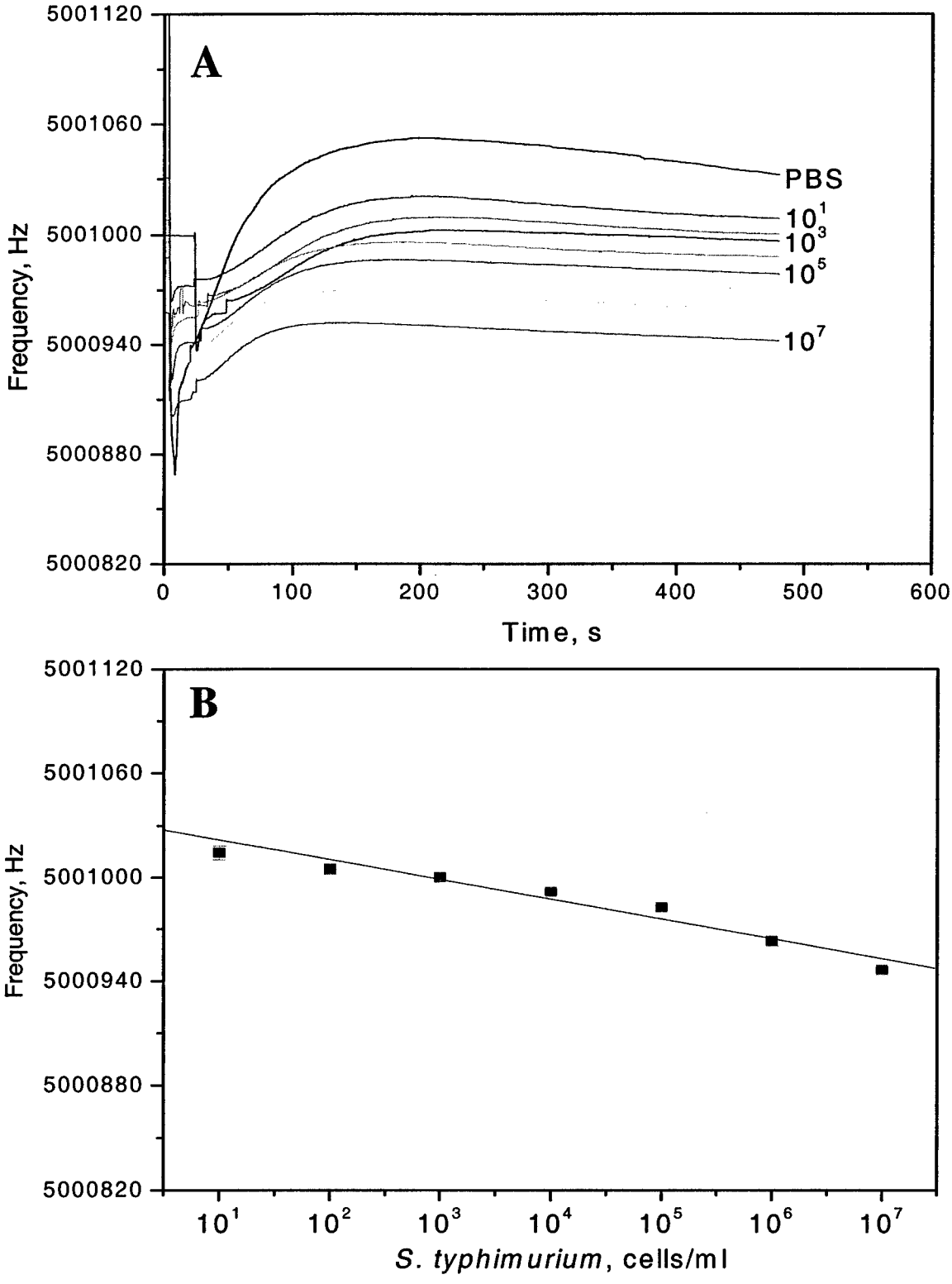
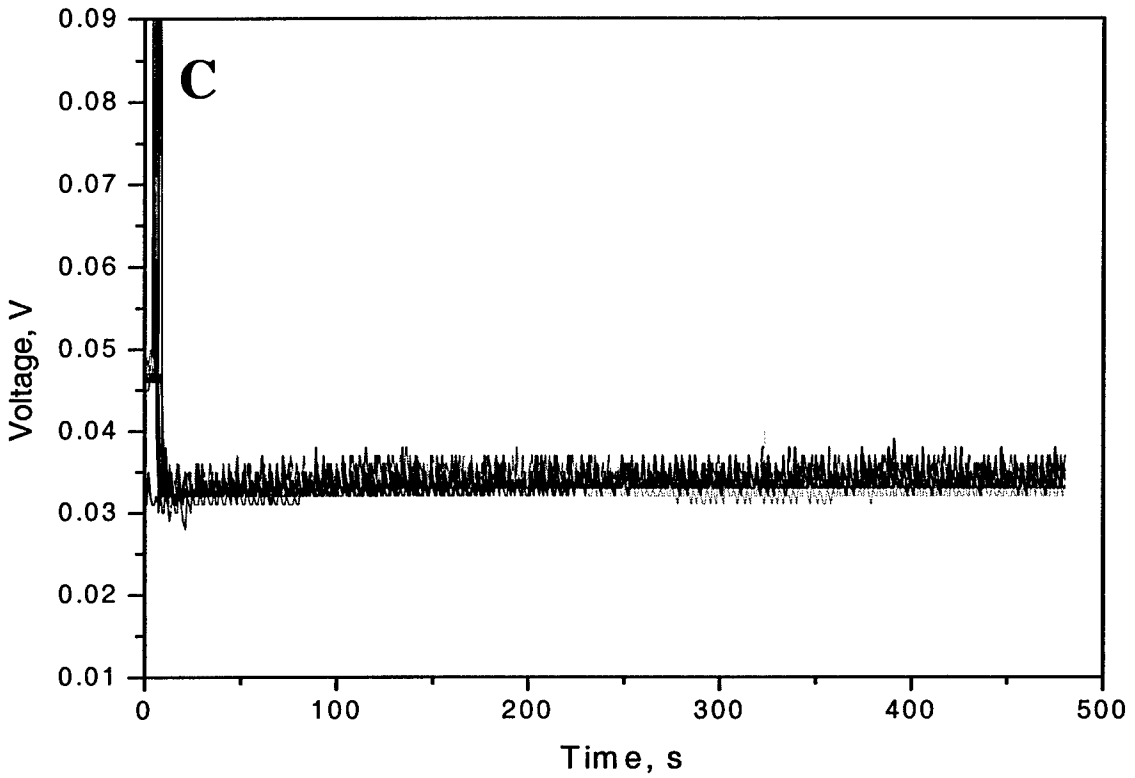


Figure 5C and 5D.



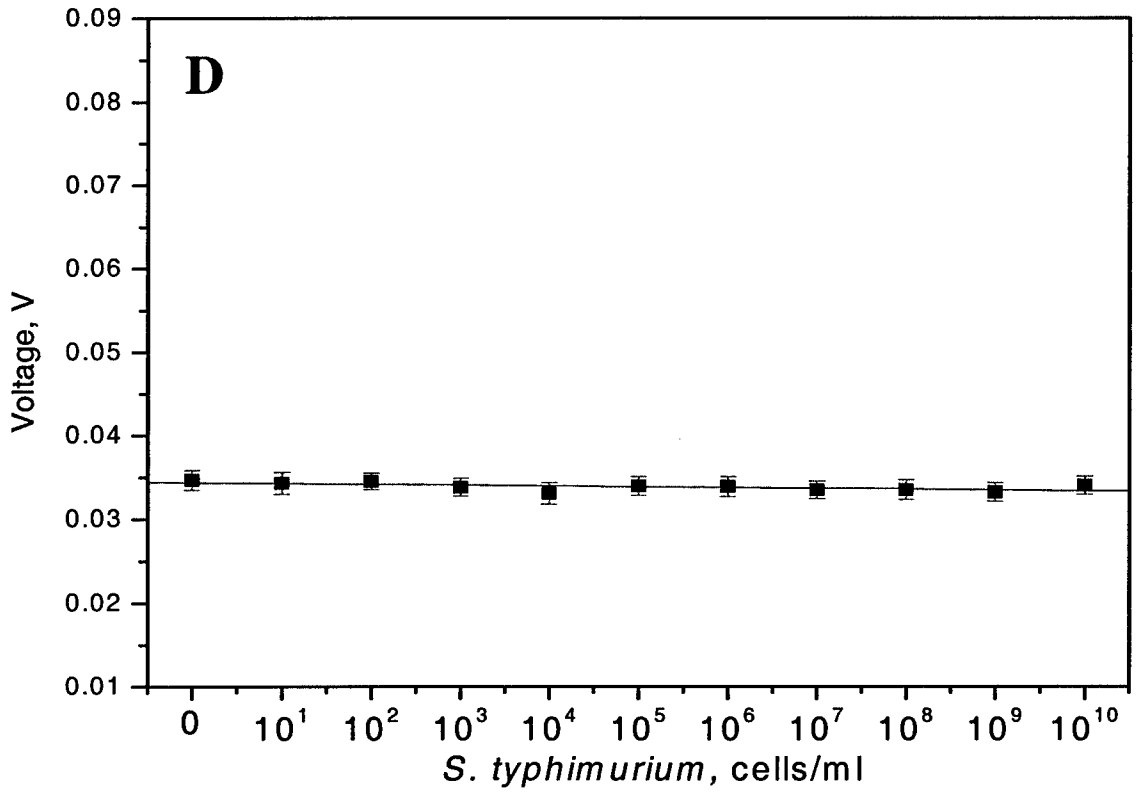


Figure 6A.

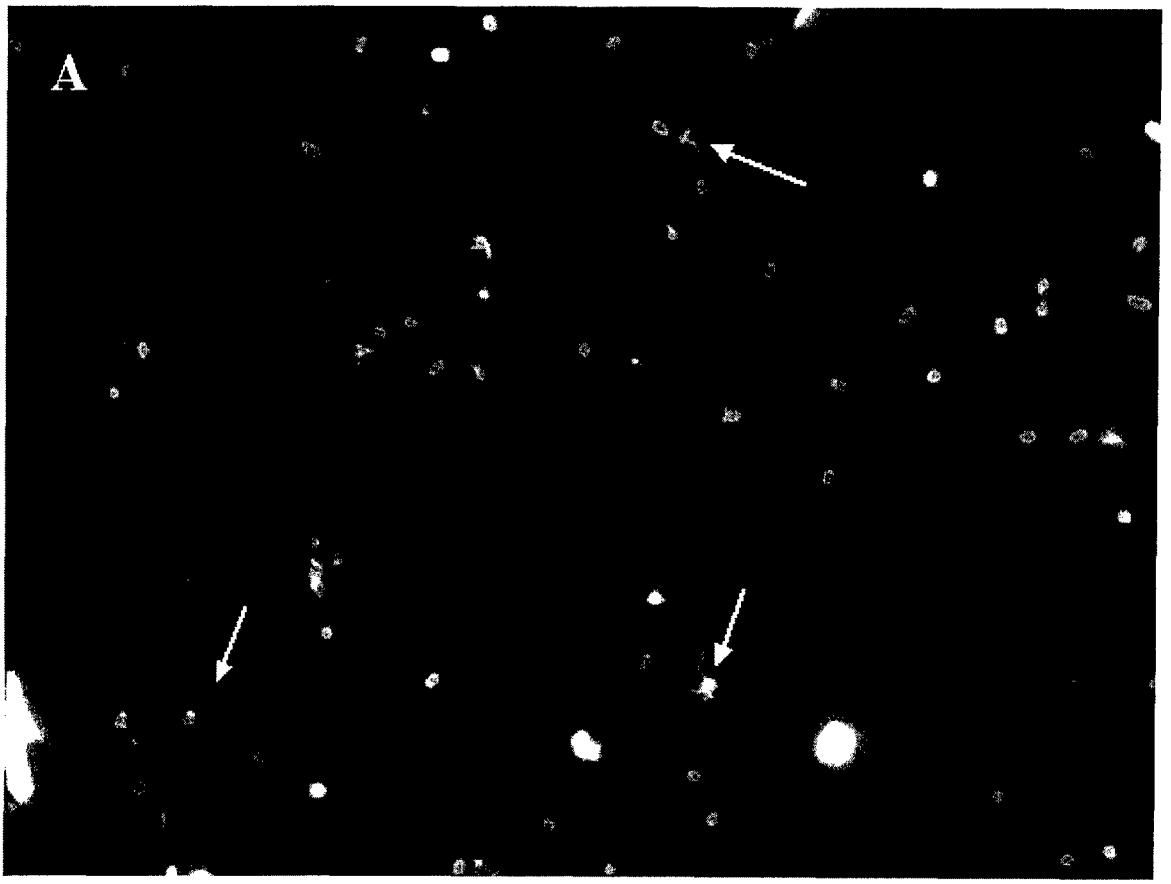


Figure 6B.

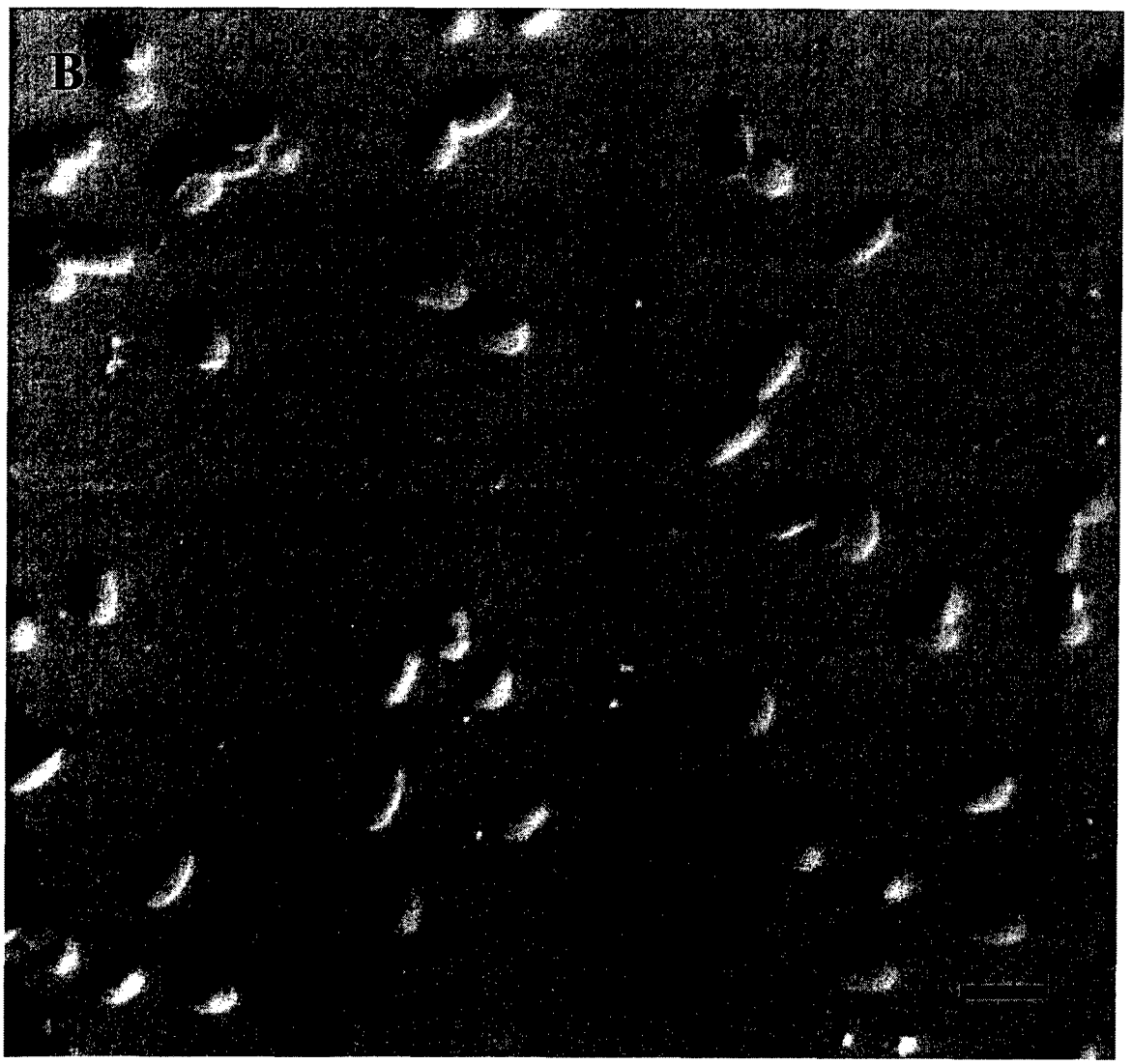


Figure 6C.

While these results typify the majority of sensor dose-responses, not all prepared sensors functioned in this manner. Some sensors had flat dose-responses, exhibiting only an initial negative frequency change when challenged with the lowest concentrations of bacteria in comparison to the control solution, PBS. This may suggest that saturation of the bioreceptor by the bacteria occurred early in the testing process. Possibly, only a small amount of phage was actively adsorbed onto the sensor electrode and/or possessed unfavorable spatial orientation as a result of the somewhat uncontrolled phage adsorption process, resulting in a small number of firm binding sites for the bacterial receptor. As well, a minority of sensors demonstrated distinct inverse correlation as previously noted with environmentally aged antibody sensors for *S. typhimurium*, and sensors prepared for *E. coli* using H-antigen antibodies (Olsen et al., 2003). Again, we hypothesize that the nature of the deposition process results in less than optimal spatial orderliness of phage on the sensor. This in turn results in loose binding of the analyte at the solid/liquid interface. We are currently researching the effects that tethered macromolecules have on resonator frequency and anticipate releasing these results in the near future to show loose binding as a mass-spring system based on the research of Dybwad (1985) and others. We also hope to identify the specific bacterial receptor targeted by the phage, which would provide great insight into loose binding if the receptor is an outer membrane associated structure such as flagella or fimbriae (Sorokulova et al., 2005).

4. Conclusions

The results of this research demonstrate developmental proof-of-concept biosensors for *S. typhimurium* in aqueous samples, based on affinity-selected recombinant filamentous phage probes. Sensors prepared with phage as probes could be an effective analytical method for detecting and monitoring quantitative changes of bacterial agents under any conditions that warrant their recognition, including food products and possibly biological warfare applications. Furthermore, the nature of the bioreceptor layer holds potential utilization for development against any bacteria, virus or toxin to which a corresponding phage could be affinity selected for. Therefore, other potential markets include clinical-based diagnostics, research, and industrial use.

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Figure Legends

Fig. 1. (A) Representative graph depicting frequency change as a function of phage adsorption to resonator over time. Eighteen-hour incubation period is shown. f_S: application of phage in suspension to clean, dry resonator at steady state: 5,012,338 ± 0.5 Hz. f_R: removal of phage suspension, washing, and air-drying of resonator. f_E: dried resonator at steady-state: 5,012,177 ± 0.5 Hz. $\Delta f = (f_S) - (f_E) = -161 \pm 0.5$ Hz. (B) Representative graph of a clean resonator with degassed water only (control) depicting frequency change as a function of time. One-hour incubation period is shown. f_S: application of water to clean, dry resonator at steady state: 5,000,167 ± 0.5 Hz. f_R: removal of water and air-drying of resonator. f_E: dried resonator at steady-state: 5,000,167 ± 0.5 Hz. $\Delta f = (f_S) - (f_E) = 0 \pm 0.5$ Hz.

Fig. 2. Conglomerate data from timed deposition studies (Table 1). Fitted sigmoidal curve indicates a strong relationship ($R^2 = 0.98$) between time and frequency change as a result of phage adsorbing to resonators. Bars are SD.

Fig. 3. Fluorescence microscopy of operational sensor undergoing phage deposition. (A) Clean resonator (control) operating in real-time. Arrow indicates edge of sensing electrode. Magnification, × 1000. (B) Same resonator following phage deposition for 1 h and washing with PBS. Field of view encompasses active area of the sensing electrode. The size differential of phage "bundles" is easily discernable. Magnification, × 1000.

Fig. 4. (A) Frequency responses of phage biosensor to differing concentrations of *S. typhimurium* as a function of time. Concentrations ranged from 0 (PBS) to 10^7 cells/ml (select values shown to right of response lines). (B) Dose-response relation of mean values (n = 2800 ± 2) of steady-state output sensor frequencies as a function of *S. typhimurium* concentration. Bars are SD = 2.9 – 10.0 Hz. Curve is linear least squares fit to experimental data ($R = -0.98$, slope = – 10.9 Hz, $p < 0.001$).

Fig. 5. (A) Voltage responses of clean resonator to increasing concentrations of *S. typhimurium* as a function of time. Concentrations ranged from 0 (PBS) to 10^{10} cells/ml. (B) Dose-response relation of mean values (n = 431) of steady-state output sensor voltages as a function of *S. typhimurium* concentration. Bars are SD. Curve is linear least squares fit to experimental data points ($R = -0.61$, slope = – 0.09 mV, $p < 0.05$). (C) Short-term phage adsorption stability. Dry resonator previously adsorbed with filamentous phage for 18 hours (f_S: 4,999,973 ± 0.5 Hz) was washed (W) five times with degassed water then air-dried (f_E: 4,999,973 ± 0.5 Hz). $\Delta f = (f_S) - (f_E) = 0 \pm 0.5$ Hz, indicating no loss of phage from active area of the sensing electrode. (D) Long-term phage adsorption stability. Dry resonator previously adsorbed with filamentous phage (f_S: 4,999,077 ± 0.5 Hz) was incubated with degassed water for 55 hours then air-dried (f_E: 4,999,077 ± 0.5 Hz). $\Delta f = (f_S) - (f_E) = 0 \pm 0.5$ Hz, again indicating no loss of phage from active area of the sensing electrode.

Fig. 6. (A) Fluorescence microscopy image of *S. typhimurium* attached to the surface of a resonator previously adsorbed with filamentous phage (discernable in background). The

majority of bacteria demonstrated rigidly attached flagella (arrows). Magnification, × 1000. (B) Scanning electron micrograph of *S. typhimurium* binding to phage immobilized to the surface of a sensor by physical adsorption. Magnification, × 3000; bar = 5 μm. (C) Scanning electron micrograph of control sensor with only phage physically adsorbed. The smooth surface is indicative of a polished resonator. Phage resolution was not possible. Magnification, × 1000; (black) bar = 10 μm.